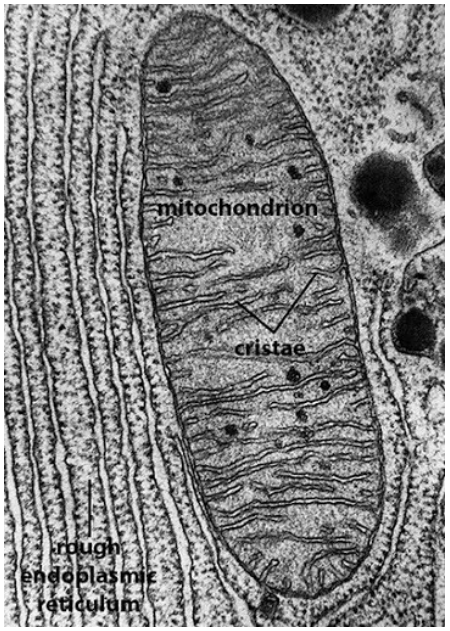
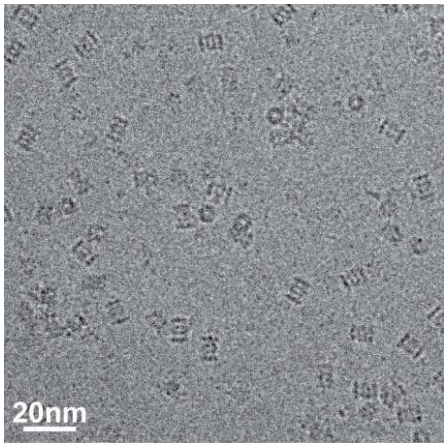
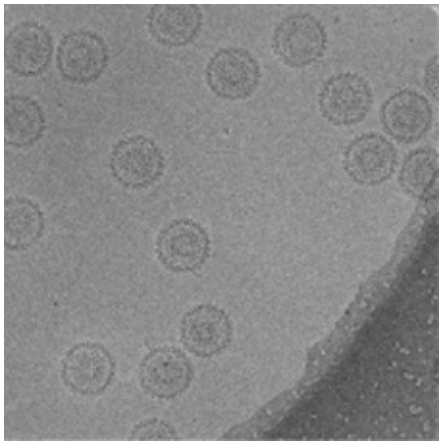
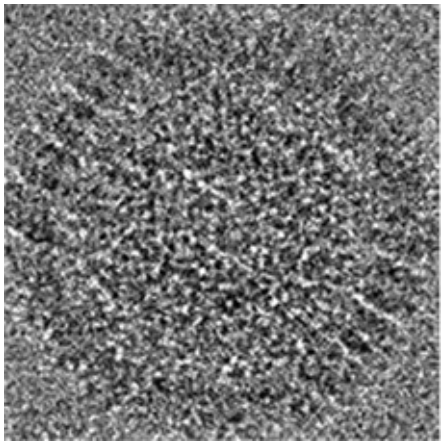
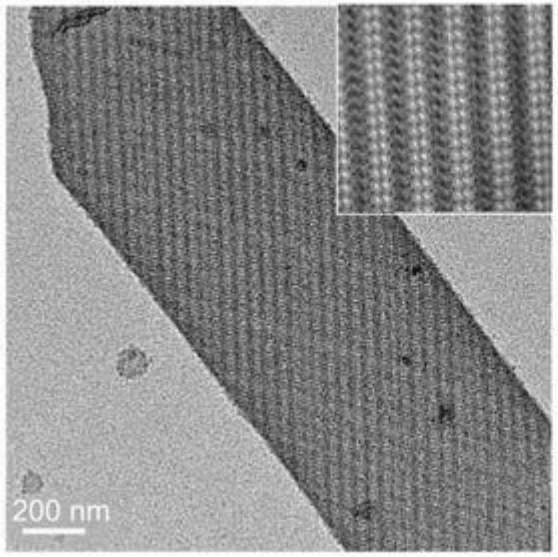
Electron Microscopy

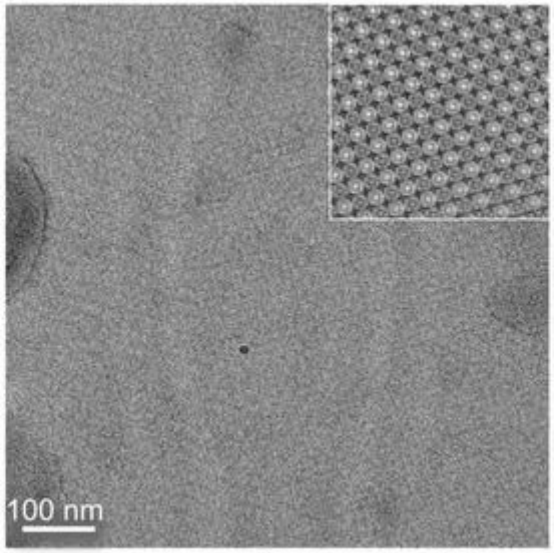


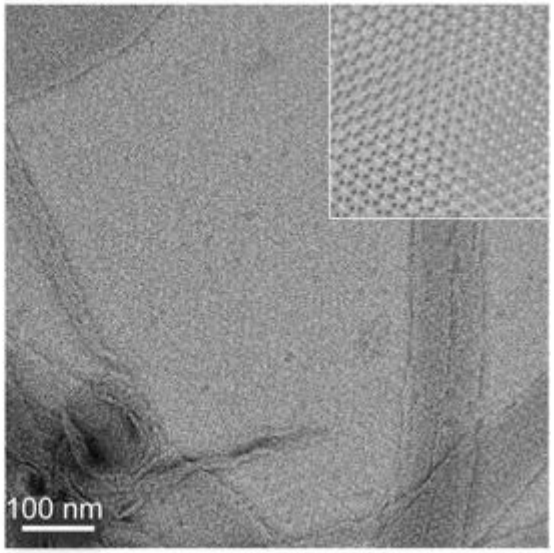












Corso di Biocristallografia e Microscopia Elettronica

rdezorzi@units.it

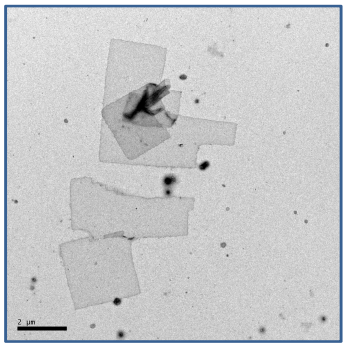
Electrons and X-rays

	ELECTRONS	X-RAY
Charge	-1.6×10^{-19} C	No charge
Mass	9.10×10^{-31} kg	No mass
Wavelength	0.02Å (at 300kV)	Wavelength of ~1Å
Interaction	Stronger	Weaker
Scattering power	Electrons: ~10^5 x scattering power of X-ray	
Sample	From single proteins (>150kDa) to small 2D crystals (<0.1µm), to organelles… and 3D nanocrystals??	From single proteins to 3D crystals (>1µm)

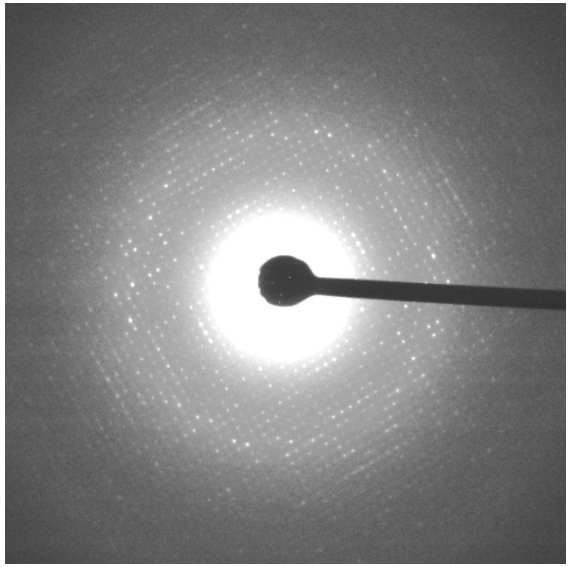
Bragg:
$2d \cdot sin\theta = \lambda$

Overview of EM techniques in Structural Biology

Imaging



Diffraction



2D crystals → **Electron crystallography**

Protein in solution → **Single particle EM**

3D crystals → **Electron crystallography on 3D crystals**

Organelles or big proteins in solution → **Electron tomography**

Overview of EM techniques in Structural Biology

2D crystals → Electron crystallography

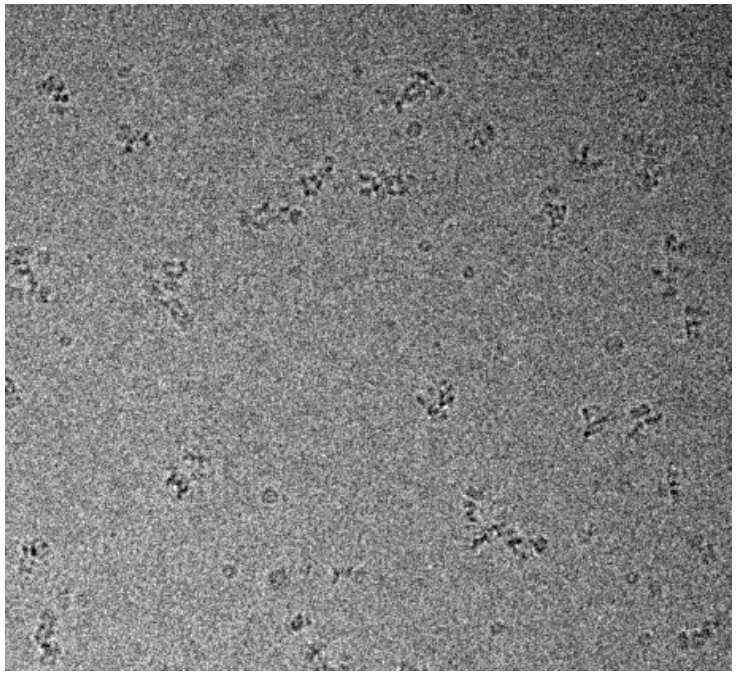
Protein in solution → Single particle EM

3D crystals → Electron crystallography on 3D crystals

Organelles or big proteins in solution → Electron tomography

Negative staining

Cryo



Overview of EM techniques in Structural Biology

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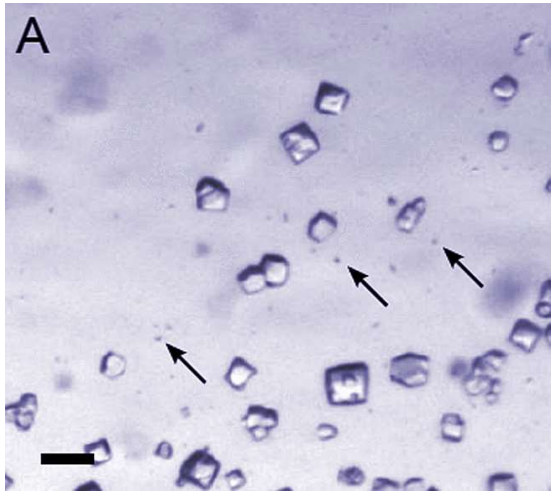

Overview of EM techniques in Structural Biology

2D crystals → **Electron crystallography**

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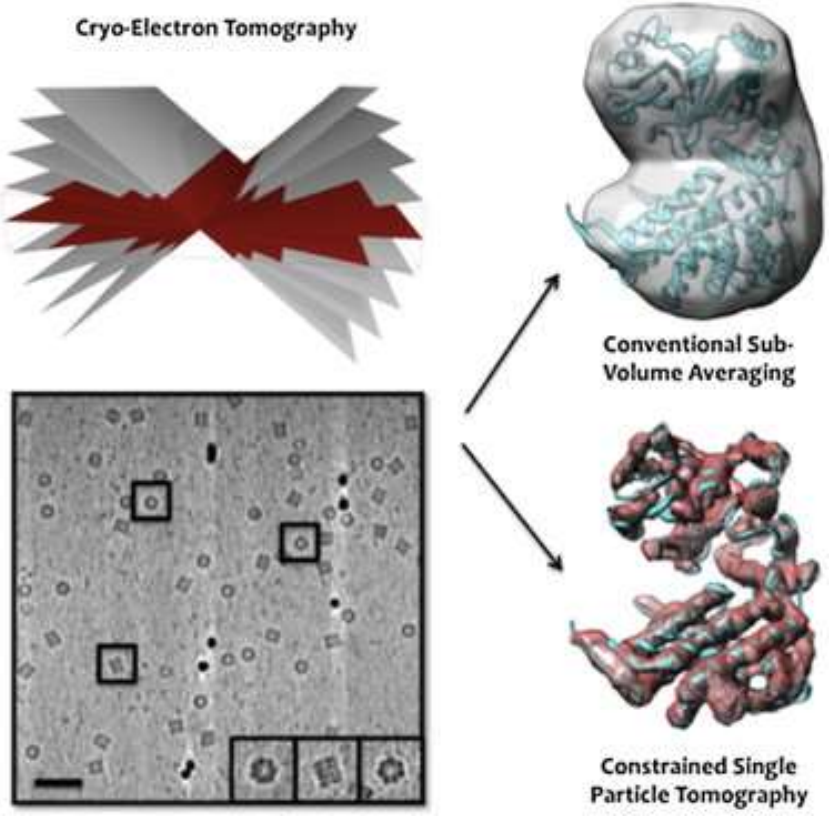
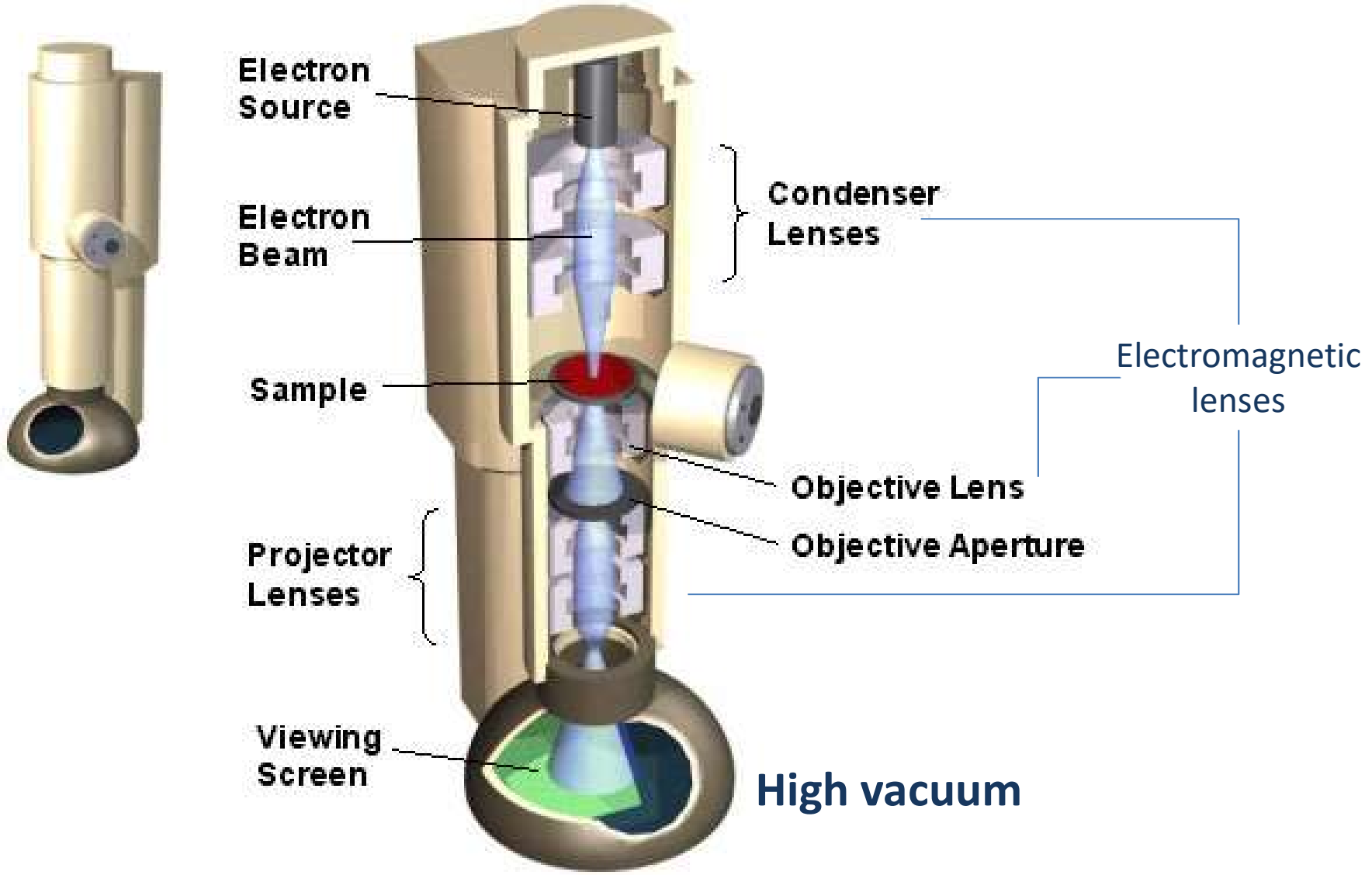
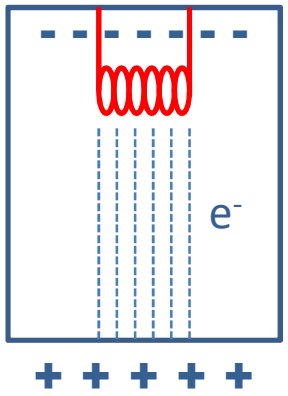


Transmission Electron Microscope



Electron gun

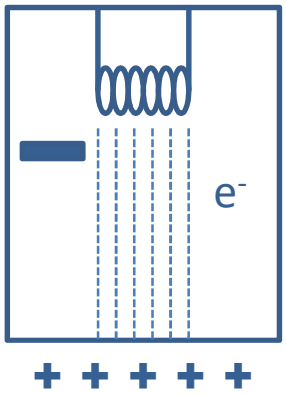
Thermionic: W or LaB_6
Source heated above extraction energy of electrons of the material. Electrons accelerated by electric field



Easier to exchange, less expensive

Field Emission Gun (FEG): W filament
Emission obtained by an electrode at ~2 kV that tunnels out electrons through narrow potential barrier

More spatial and temporal coherence, higher intensity



$$E = e \cdot V = \frac{h^2}{2m\lambda^2}$$

E: electron energy
e: electron charge
V: accelerating voltage (100-300 kV)
h: Planck's constant
m: relativistic mass of the electron
λ: electron wavelength

Coherence: required to obtain high resolution data. Spatial coherence is optimal when beam is collimated (parallel illumination); temporal coherence depends on wavelength spread of the incident electrons.

Brightness: number of electrons that pass through an area per second, per steradian. Thermionic source: 10^6-10^7 A/(cm^{2}rad^2). FEG: 10^7-10^8 A/(cm^{2}rad^2).

TF20 FEI

Titan Krios FEI

Accelerating Voltage

80kV → Energy → 300kV

Wavelength ←

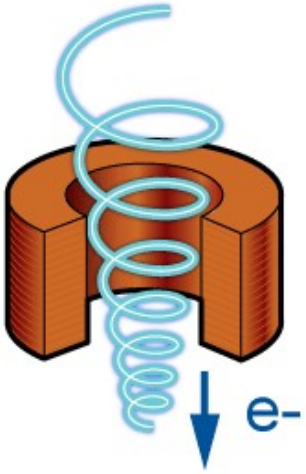
Strength of interaction ←

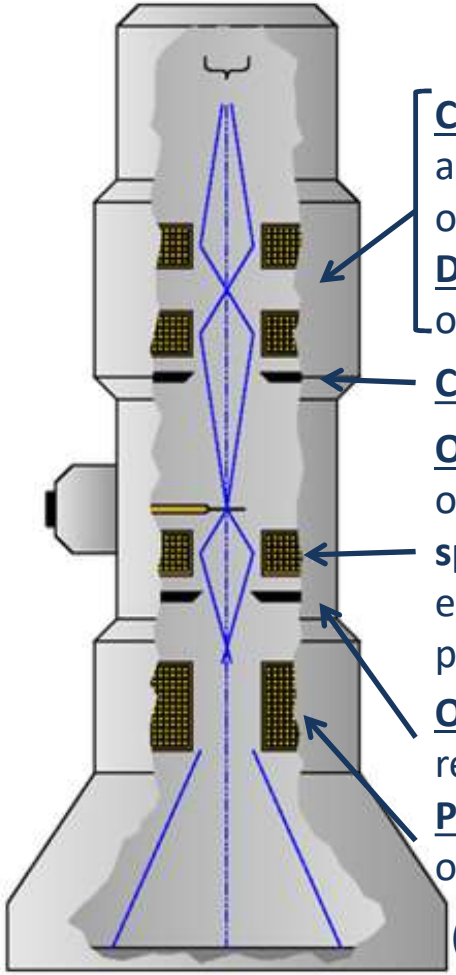
Radiation damage ←

Contrast ←

Magnetic lenses

Magnetic fields that induce spiral movement of electrons.
Changing current in the lens changes focal length.
Alignment of the lenses (and apertures) before data collection: ≈ 40 min/day





Condenser lenses: collimate and focus the beam. Allows to change beam size and intensity: it affects how parallel the illumination is. Require alignment to obtain better **spatial coherence**.

Deflection coils: to position the beam and align it with the optical axis of the objective lens.

Condenser aperture: change intensity. Requires alignment.

Objective lens: focusing of the image. Changing focal length (focus) of the objective lens changes phase of the wave function. Objective lens introduces **spherical and chromatic aberrations** and can generate **astigmatism**. Images in electron microscopy are usually collected in **defocus**. Objective lens is positioned very close to the sample, to minimize aberration effects.

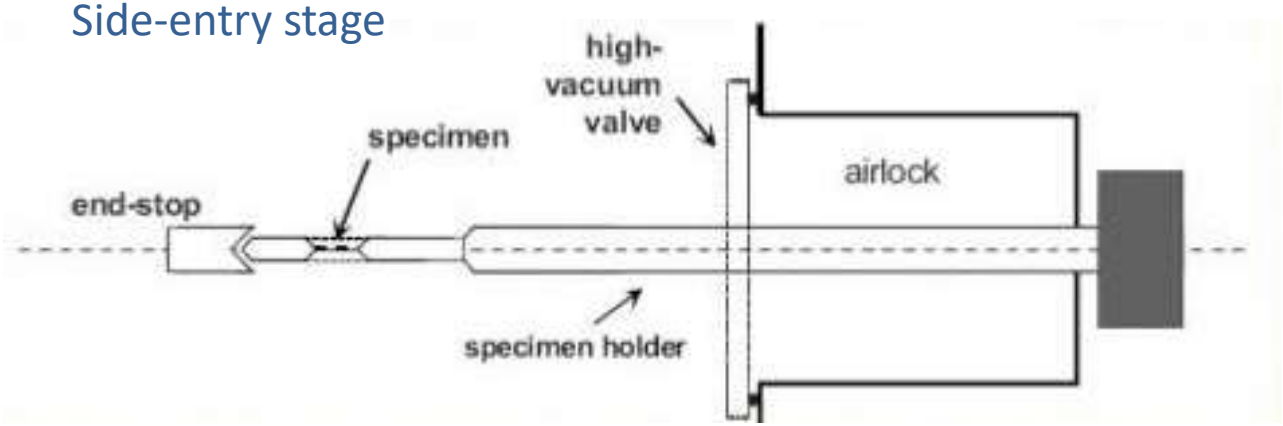
Objective aperture: improves **contrast**, but can also limit achievable resolution of the image.

Projector lenses system: generate **magnification** of the object to be projected on the screen/camera.

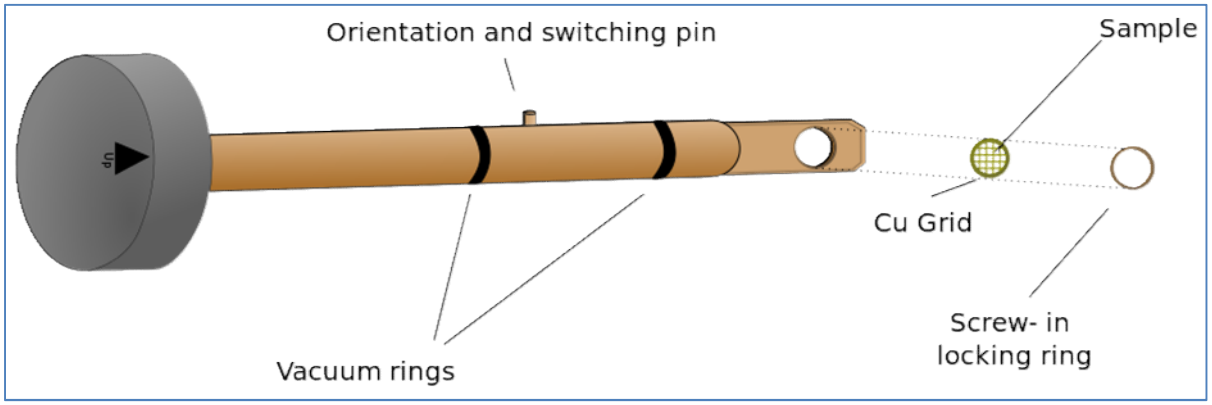
(Eventually) **Energy filter lenses**.

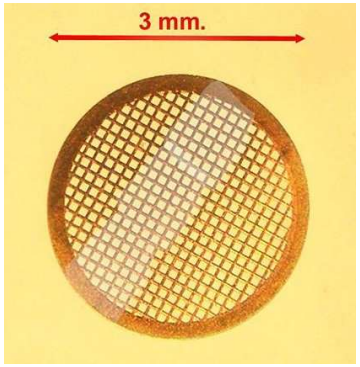
Specimen stage and sample holders

Side-entry stage



Sample holders for negatively stained samples





Sample holders for Cryo samples

Detectors

For alignment and sample evaluation (not recording!):

FLUORESCENT SCREEN or TV CAMERA

For data collection:

PHOTOGRAPHIC FILM CAMERA or SOLID STATE CCD CAMERA

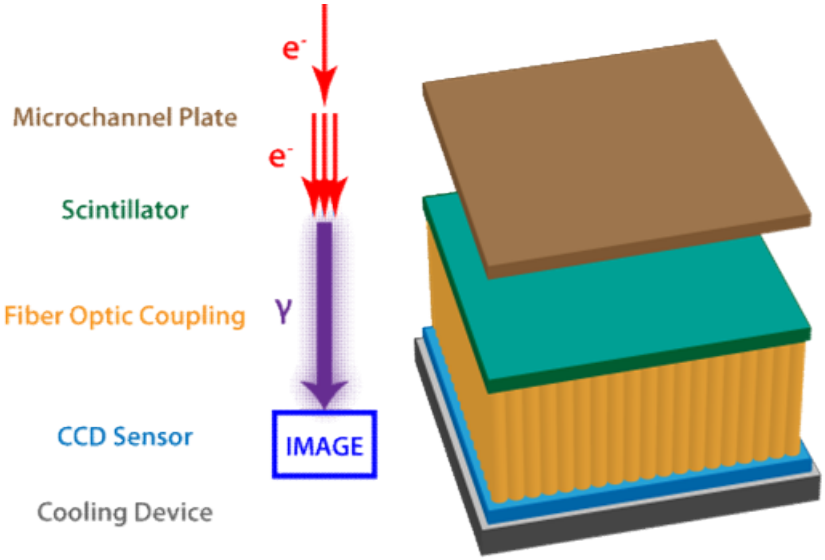
- **Area of the detector**: number of pixels of the image that can be recorded
- **Pixel size**: connected to the Nyquist limit (maximum resolution that can be obtained)
- **Signal-to-Noise Ratio (SNR)**: noise generated (1) by the inelastically scattered electrons and (2) from the detector readout
- **Detector Quantum Efficency (DQE)**: $DQE = \frac{SNR_{readout}{}^2}{SNR_{input}{}^2}$

DQE depends on exposure level, saturation effects, accelerating voltage… Typical values of DQE

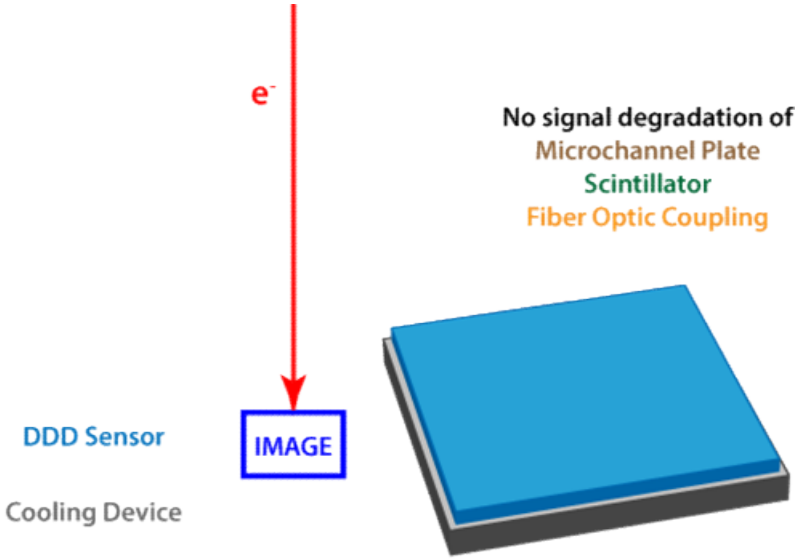
(for imaging): 0.6 for films; 0.7-0.8 for CCD cameras.

- **Dynamic range**: number of grey levels that can be separated before reaching the saturation level. For films: 100; for CCD cameras: 10^4.

Direct Detection Devices (or DDD camera)



MCP + CCD

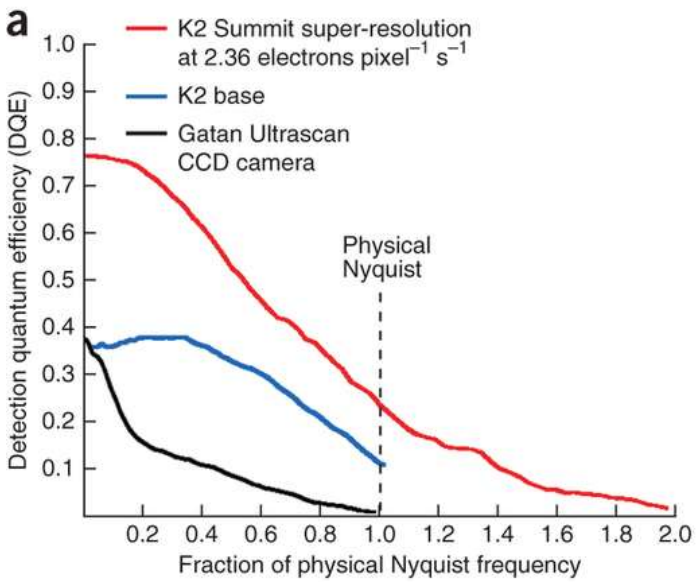


Direct Detection Camera

NEW

- Direct Detection Devices: direct detection of electrons with no light conversion

Revolution in the field!!

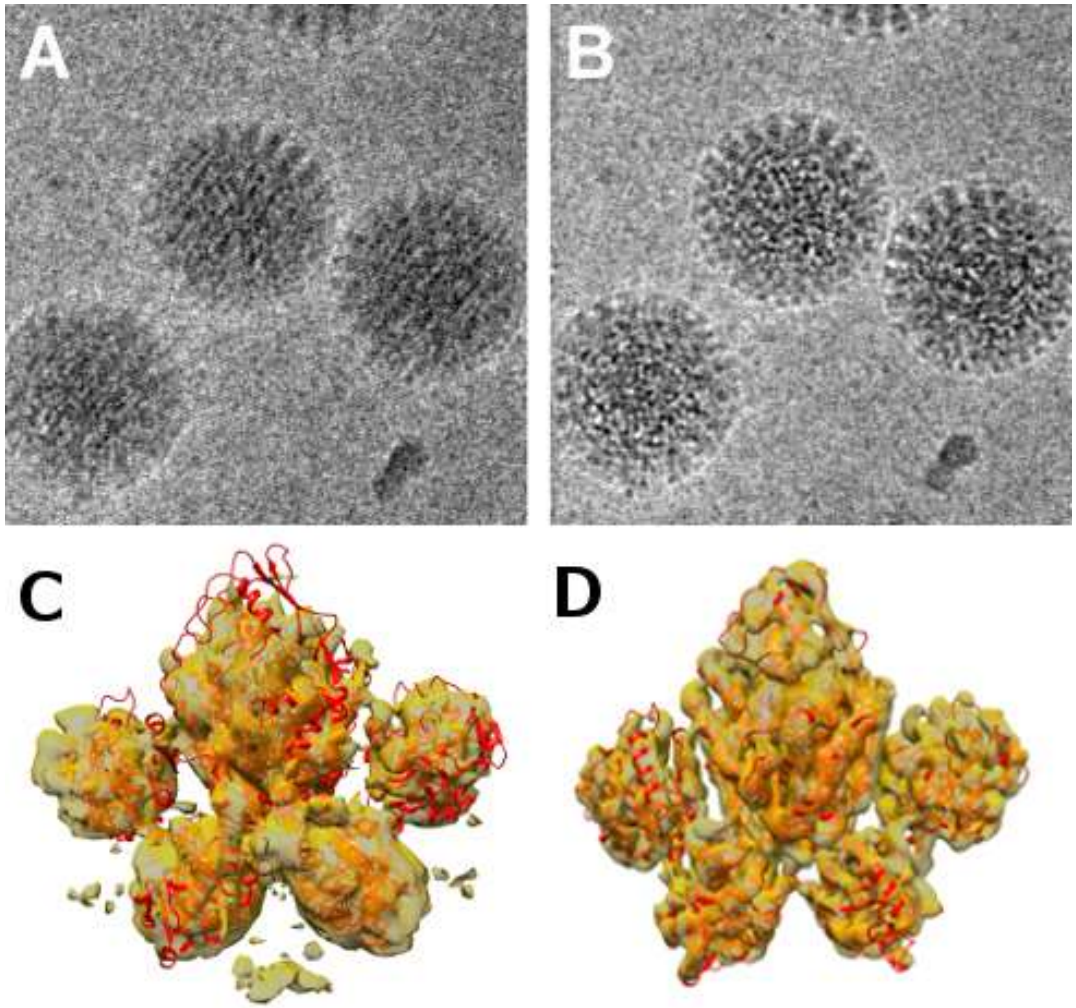


Direct Detection Devices (or DDD camera)

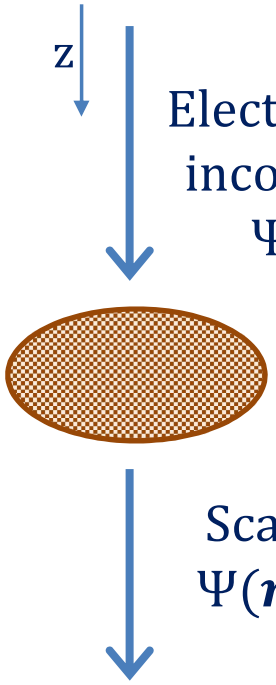
✓More sensitive — Lower dose & less radiation damage!

✓Less noise — Higher SNR, higher resolution!

✓Fast readout — Motion correction on frames, higher resolution!



Bright field electron microscopy



Electron beam as incoming wave: $\Psi_0 = e^{ikz}$

Scattered wave: $\Psi(\boldsymbol{r}) = \Psi_0 e^{i\phi(r)}$

INFORMATION

Elastic scattering

No energy loss
Important at high resolution

\+

NOISE

Inelastic scattering

Wavelenght change
Significant at low resolution

Interaction of the incident beam with the **Coulomb potential** C of the object causes a **phase shift** ϕ:

$$\phi(\boldsymbol{r}) = \int C(\boldsymbol{r}, z)\, dz$$

In the **weak phase object approximation**, the phase shift is considered small compared to the unscattered wave. With a series expansion, the scattered wave expression is:

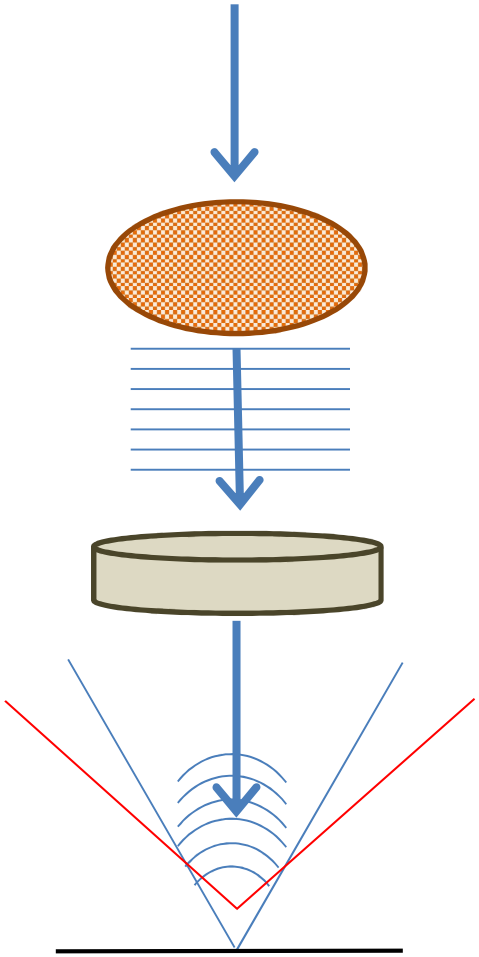
$$\Psi(\boldsymbol{r}) = \Psi_0 [1 + i\phi(\boldsymbol{r}) - \frac{1}{2}\phi(\boldsymbol{r})^2 + \cdots]$$

Unscattered wave

Scattered, 90° phase retarded wave

Smaller, higher order components

Effect of the objective lens



The presence of the objective lens transforms the plane wave in a spherical wave, focused in the back focal plane.

Due to interference of the parallel waves, in the back focal plane, distant from the object, the wave function can be written as the Fourier Transform of the scattered wave:

$$\Psi_{bf}(\boldsymbol{s}) = FT[\Psi(\boldsymbol{r})]$$

with **s** scattering vector.

However, the presence of the lens introduces deformations in this wave function:

$$\boxed{\Psi_{bf}(\boldsymbol{s}) = FT[\Psi(\boldsymbol{r})]\exp[i\gamma(s)]}$$

The $\gamma(s)$ term includes (1) a component due to **defocus** Δz (and **astigmatism** z_a in the direction α_0) and (2) a component of **spherical aberration** C_s:

$$\gamma(s,\alpha) = -\pi\lambda\left[\Delta z + \frac{z_a}{2}\sin 2(\alpha-\alpha_0)\right]s^2 + \frac{1}{4}\lambda^3 C_s s^4$$

Defocus and astigmatism

Spherical aberration

The spherical aberration induces a change in the focus length for waves at different scattering angles

Atomic scattering

The wave function in the back focal plane

$$\Psi_{bf}(\boldsymbol{s}) = FT[\Psi(\boldsymbol{r})]\exp[i\gamma(s)]$$

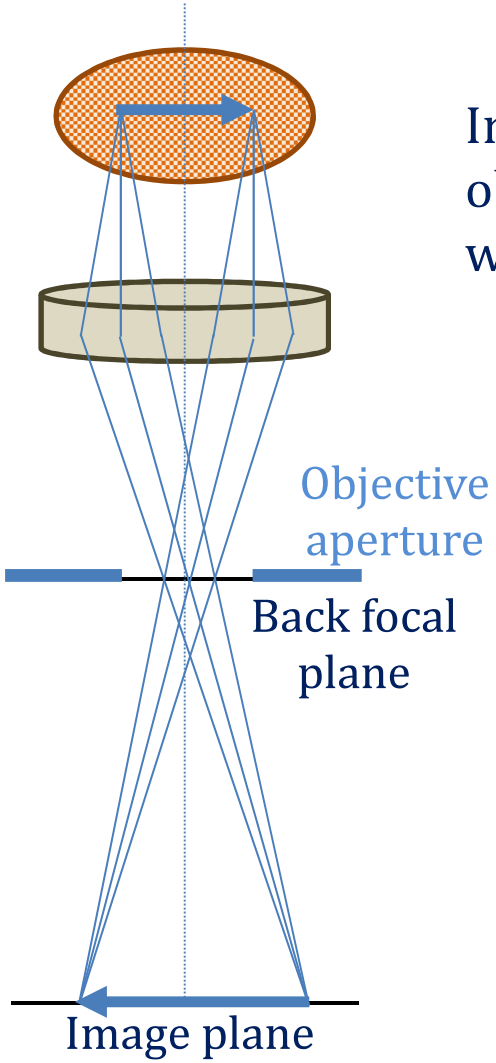
can be expressed considering the interference of scattered electrons from each atom of the specimen:

$$\Psi_{bf} = \sum_j f_j(\boldsymbol{s})\exp(-2\pi i\boldsymbol{s}\cdot\boldsymbol{r}_j)$$

Due to the strong absorption contribution, the atomic scattering factor is an angle-dependent complex number, with a real contribution and an imaginary contribution:

$$f_j(\boldsymbol{s}) = f'_j(\boldsymbol{s}) + if''_j(\boldsymbol{s})$$

Image formation

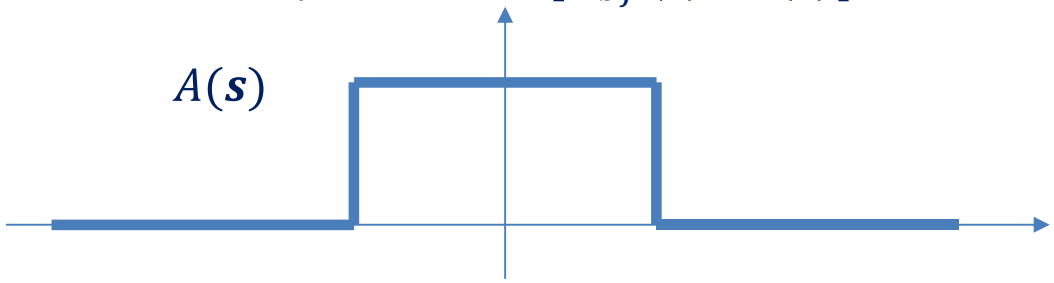


In the image plane, the formation of an image of the object is obtained by inverse Fourier Transform of the wave function in the back focal plane:

$$\Psi_i(\boldsymbol{r}) = FT^{-1}[\Psi_{bf}(\boldsymbol{s})]$$

In addition to the effects of the objective lens, the **objective aperture**, that blocks electrons scattered at high angle, causes a modification of the wave function in the back focal plane, and in the image plane:

$$\Psi_i(\boldsymbol{r}) = FT^{-1}[\Psi_{bf}(\boldsymbol{s}) \cdot A(\boldsymbol{s})]$$



The objective aperture limits the resolution of the final image (high scattering angle → high resolution), but reduces the noise and introduces **amplitude contrast** in the image.

Image:

$$I(\boldsymbol{r}) = [\Psi_i(\boldsymbol{r})]^2$$

Contrast Transfer Function

Given the object, its image $I(\boldsymbol{r})$ can be expressed by a convolution of the real object $O(\boldsymbol{r})$ and a deformation function, the **point spread function** $h(\boldsymbol{r})$:

$$I(\boldsymbol{r}) = O(\boldsymbol{r}) \otimes h(\boldsymbol{r})$$

The Fourier transform of the point spread function represents the deformation of the image that occurs in the back focal plane:

$$H(\boldsymbol{s}) = FT[h(\boldsymbol{r})] = A(\boldsymbol{s}) \sin[\gamma(s)]$$

Phase Contrast Transfer Function

In addition, effects of partial **spatial and temporal coherence** of the source introduce a decreasing exponential envelope function $E(\boldsymbol{s})$:

$$H(\boldsymbol{s}) = FT[h(\boldsymbol{r})] = A(\boldsymbol{s}) \sin[\gamma(s)]\, E(\boldsymbol{s})$$

with $E(\boldsymbol{s})$ depending on wavelength λ, defocus Δz, spherical aberration C_s (and astigmatism, if present).

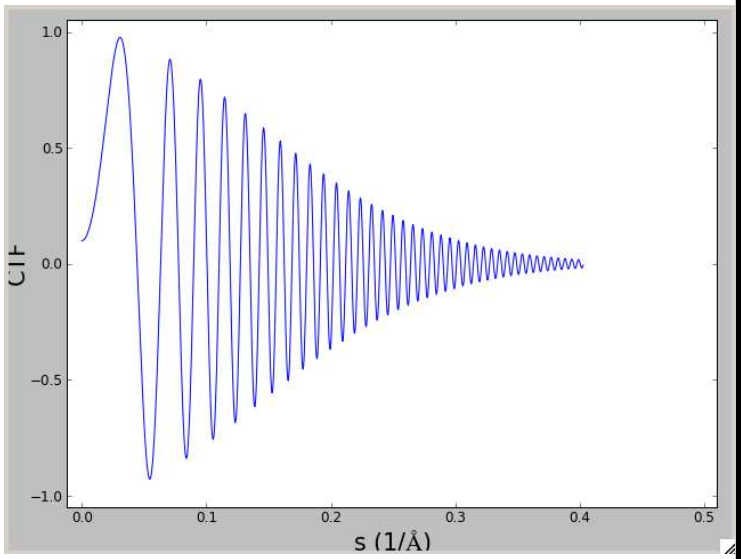
To obtain the initial object, it is necessary to determine the $H(\boldsymbol{s})$ function, or **Contrast Transfer Function (CTF).**

Contrast Transfer Function

$$H(\boldsymbol{s}) = A(\boldsymbol{s})\sin[\gamma(s)]\,E(\boldsymbol{s})$$

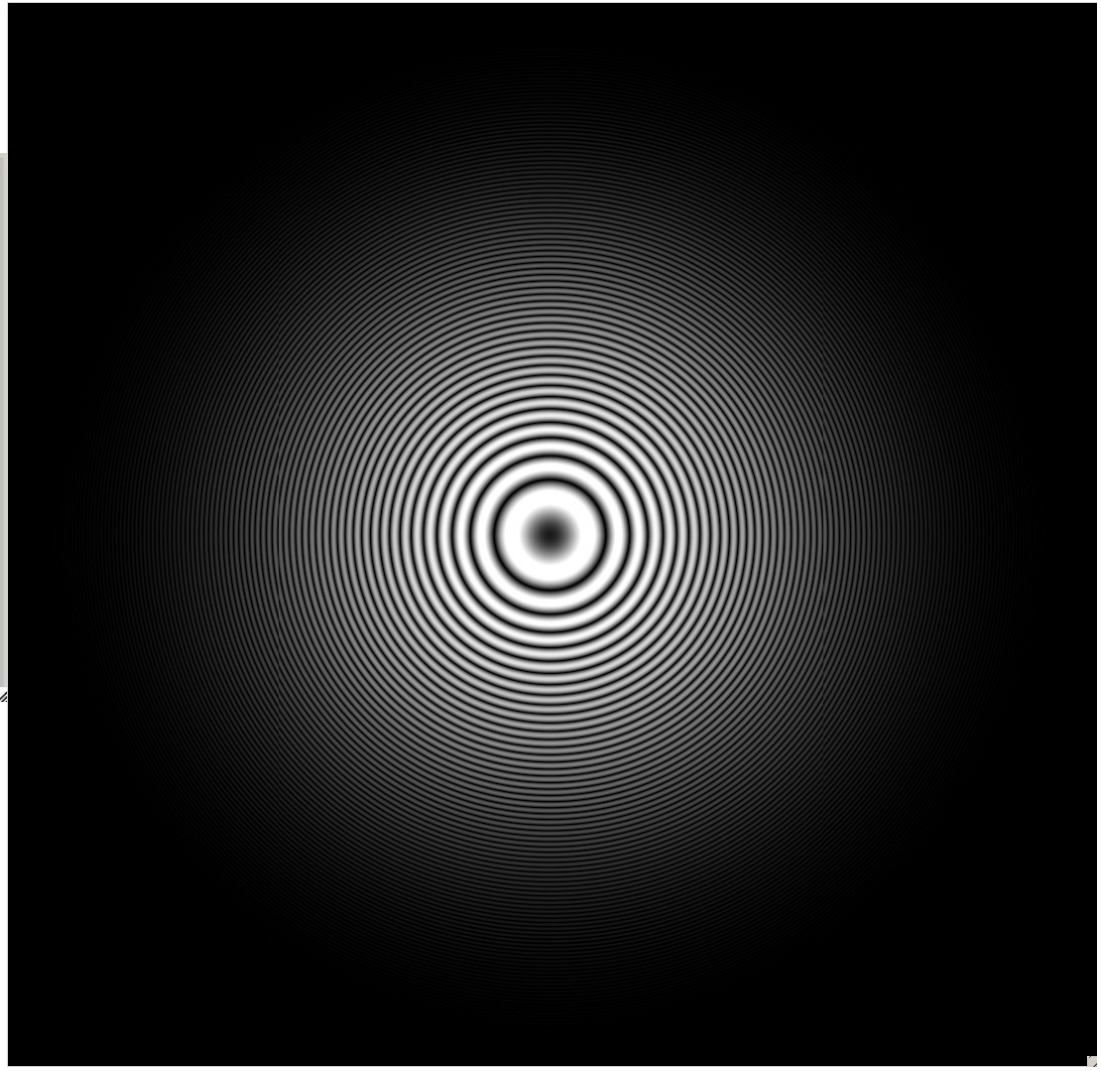
$$\gamma(s,\alpha) = -\pi\lambda\Delta z s^2 + \frac{1}{4}\lambda^3 C_s s^4$$

$$E(\boldsymbol{s},\lambda,\Delta z,C_s)$$



V=300kV
Cs=2.0mm
Δz=2.5µm
Nyquist=0.81Å^{-1}
Amplitude contrast=10%

Without astigmatism: CTF is invariant with respect to the angular direction.



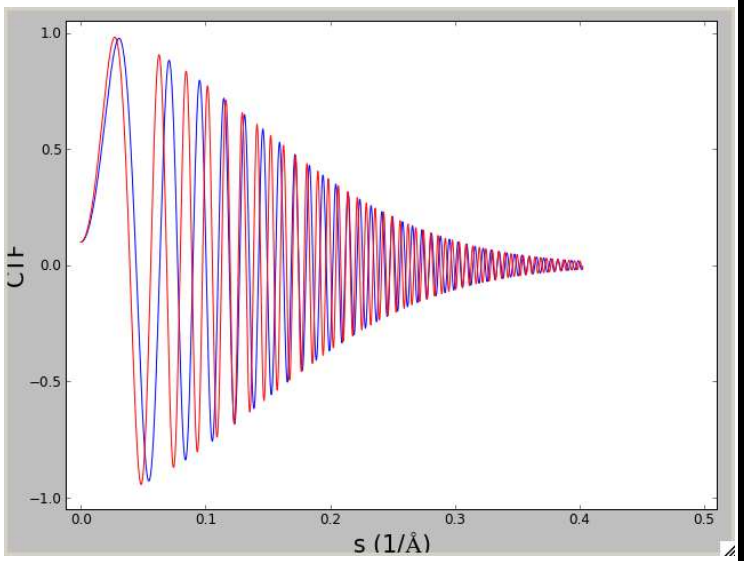
Plot and simulation: e2ctfsim.py

Contrast Transfer Function

$$H(\boldsymbol{s}) = A(\boldsymbol{s}) \sin[\gamma(s)]\, E(\boldsymbol{s})$$

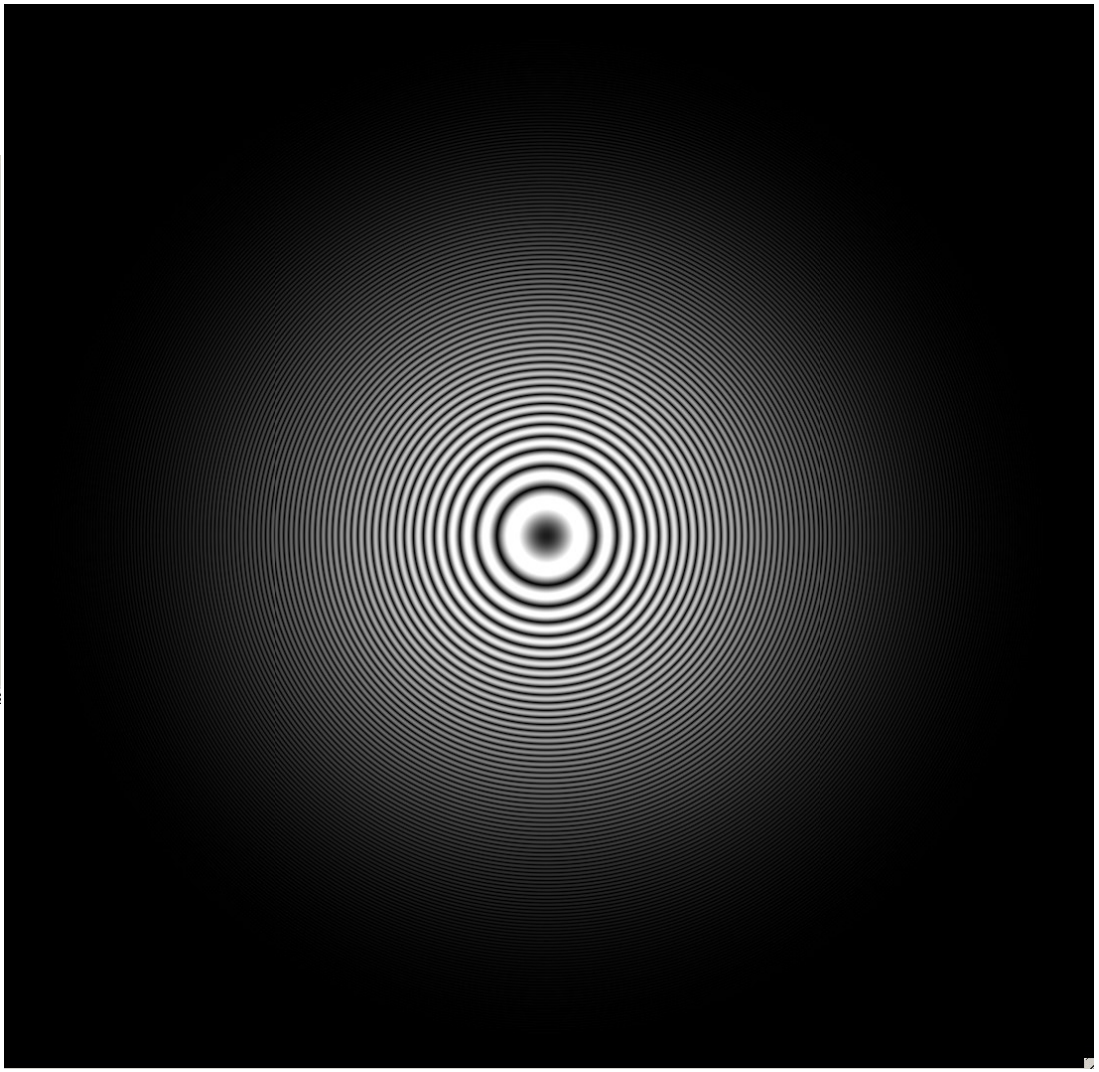
$$\gamma(s, \alpha) = -\pi\lambda\Delta z s^2 + \frac{1}{4}\lambda^3 C_s s^4$$

$$E(\boldsymbol{s}, \lambda, \Delta z, C_s)$$



V=200kV
Cs=2.0mm
Δz=2.5µm
Nyquist=0.81Å^{-1}
Amplitude contrast=10%

Without astigmatism: CTF is invariant with respect to the angular direction.



Plot and simulation: e2ctfsim.py

Contrast Transfer Function

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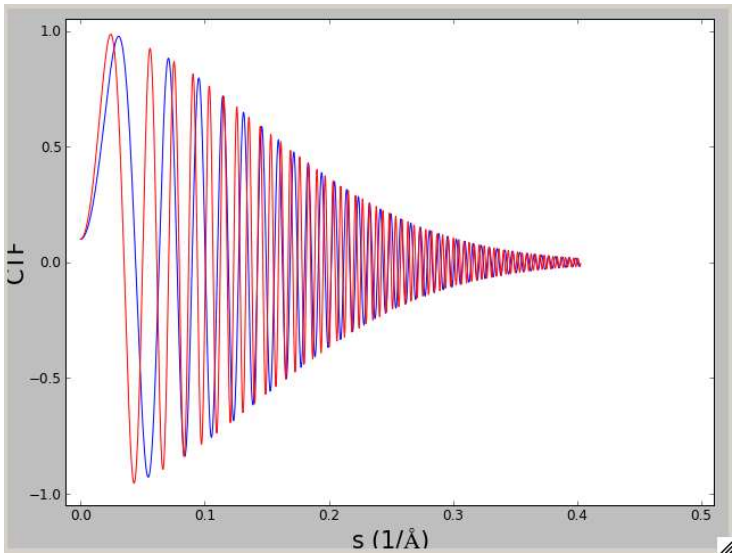
$$\gamma(s, \alpha) = -\pi\lambda\Delta z s^2 + \frac{1}{4}\lambda^3 C_s s^4$$

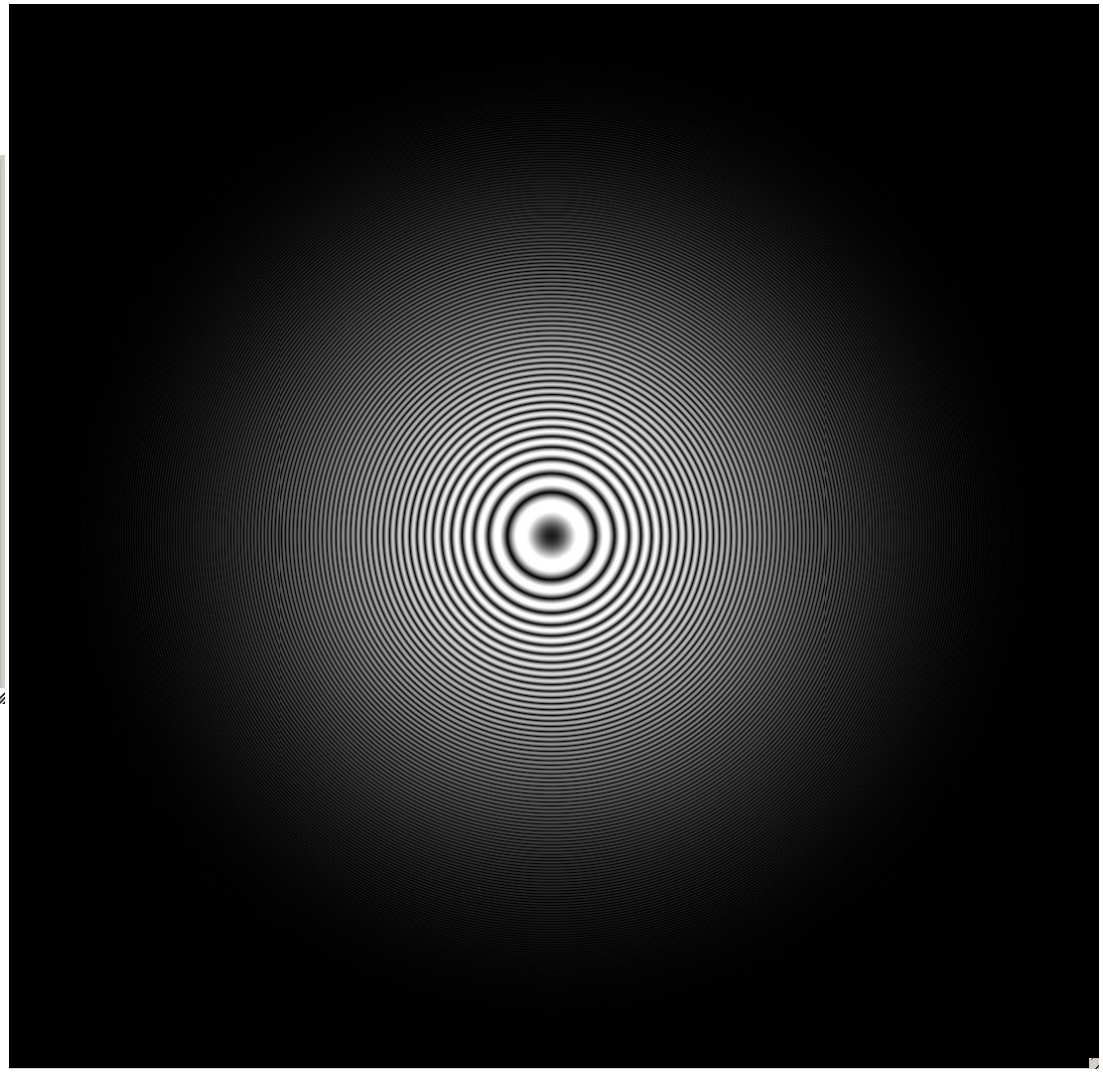
$$E(\boldsymbol{s}, \lambda, \Delta z, C_s)$$



V=300kV
Cs=2.0mm
Δz=4.0μm
Nyquist=0.81Å^{-1}
Amplitude contrast=10%

Plot and simulation: e2ctfsim.py

Without astigmatism: CTF is invariant with respect to the angular direction.

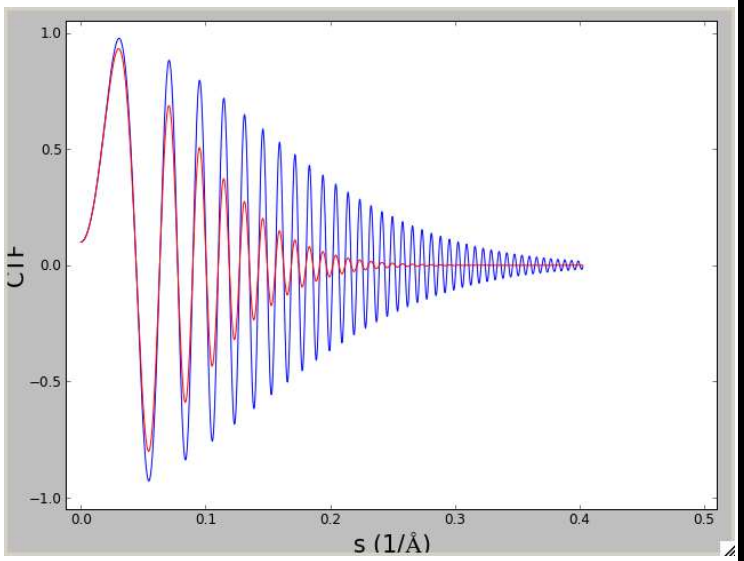


Contrast Transfer Function

$$H(\boldsymbol{s}) = A(\boldsymbol{s})\sin[\gamma(s)]\,E(\boldsymbol{s})$$

$$\gamma(s,\alpha) = -\pi\lambda\Delta z s^2 + \frac{1}{4}\lambda^3 C_s s^4$$

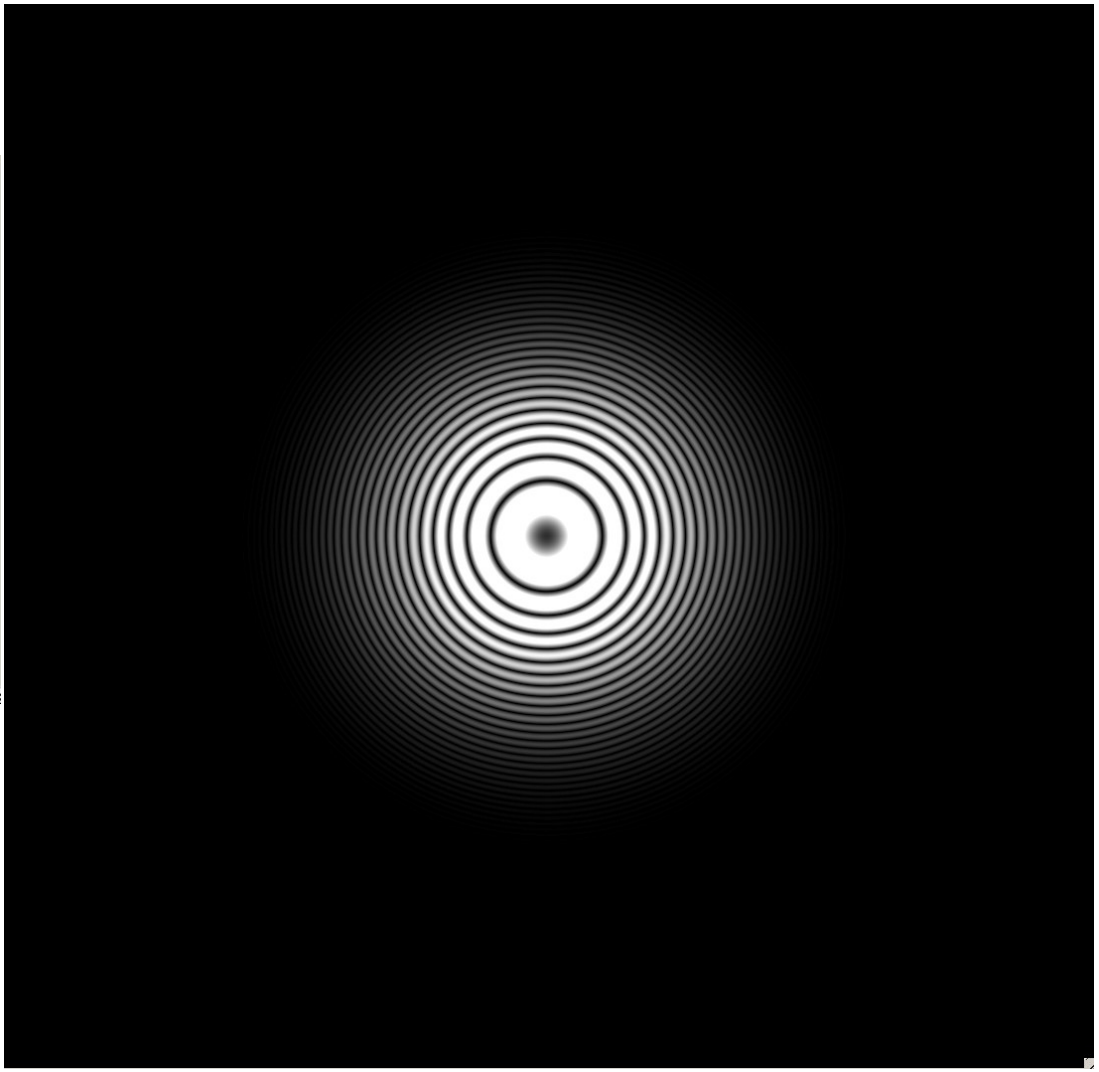
$$E(\boldsymbol{s},\lambda,\Delta z, C_s)$$



V=300kV
Cs=2.0mm
Δz=2.5µm
Nyquist=0.81Å^{-1}
Amplitude contrast=10%
Non-coherent beam

Plot and simulation: e2ctfsim.py

Without astigmatism: CTF is invariant with respect to the angular direction.



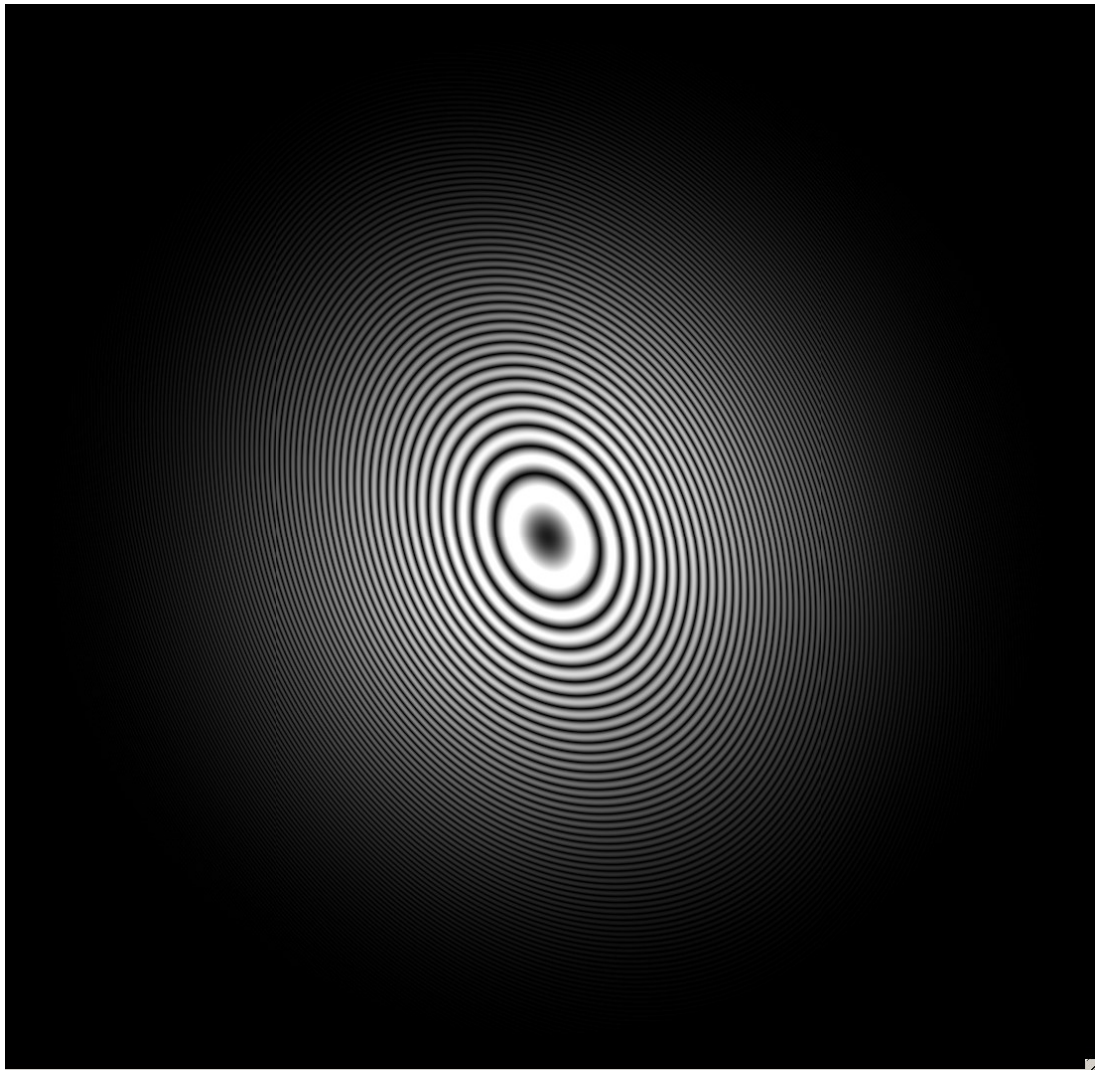
Contrast Transfer Function

$$\gamma(s,\alpha) = -\pi\lambda\left[\Delta z + \frac{z_a}{2}\sin 2(\alpha - \alpha_0)\right]s^2 + \frac{1}{4}\lambda^3 C_s s^4$$

z_a = astigmatism amplitude
α_0 = astigmatism angle

When astigmatism is present: the CTF is not rotationally symmetric.

V=300kV
Cs=2.0mm
Δz=2.5μm
z_a=1.5μm
α_0=25deg
Nyquist=0.81Å^{-1}
Amplitude contrast=10%



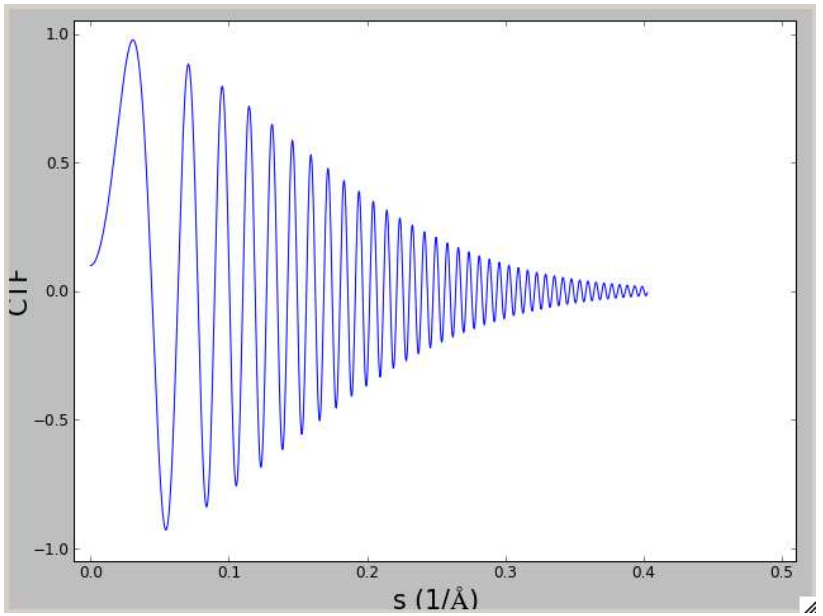
CTF correction

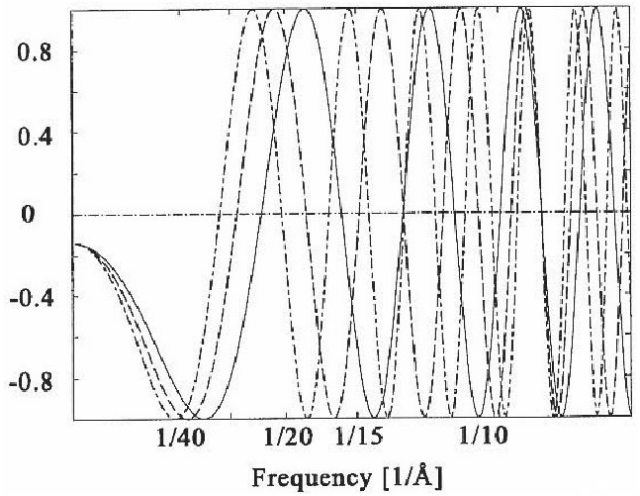
In the real space: $I(\boldsymbol{r}) = O(\boldsymbol{r}) \otimes h(\boldsymbol{r})$
In the reciprocal space: $I(\boldsymbol{s}) = H(\boldsymbol{s}) \cdot O(\boldsymbol{s})$

To obtain the real image of the object, the effect of the contrast transfer function has to be inverted.

Issue 1: *When $H(\boldsymbol{s}) = 0$, information on the sample is lost.*

Solution: When possible collect **defocus series** of images: same sample but with a different defocus value, so that information is obtained also for fequencies for which $H(\boldsymbol{s}) = 0$.



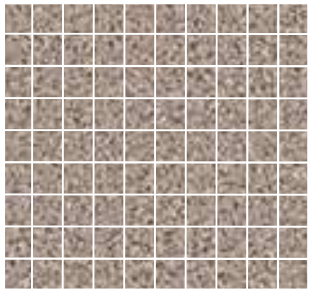


Issue 2:
Correct CTF contribution.

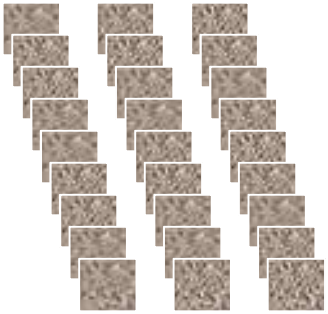
Dividing the image function by $H(\boldsymbol{s})$ would be problematic for frequencies with $H(\boldsymbol{s}) = 0$.

First determine CTF...

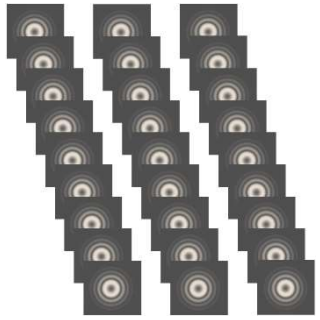
CTFFIND3



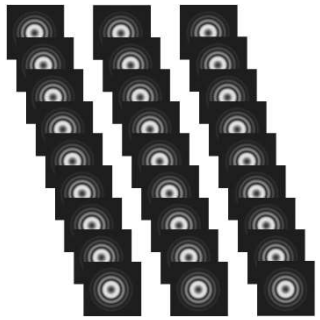
1. Divide image in tiles



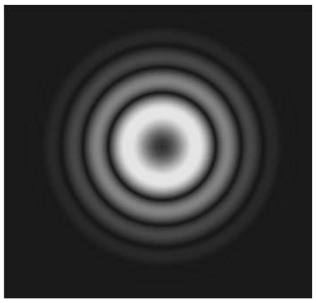
2. Remove areas with high-low pixel density variance



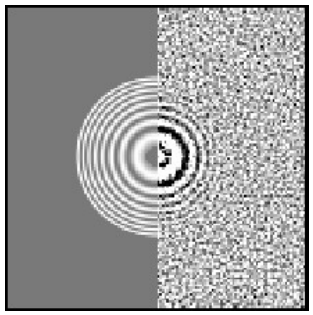
3. FFT



4. Background correction: empirically estimated, monotonically decreasing background function



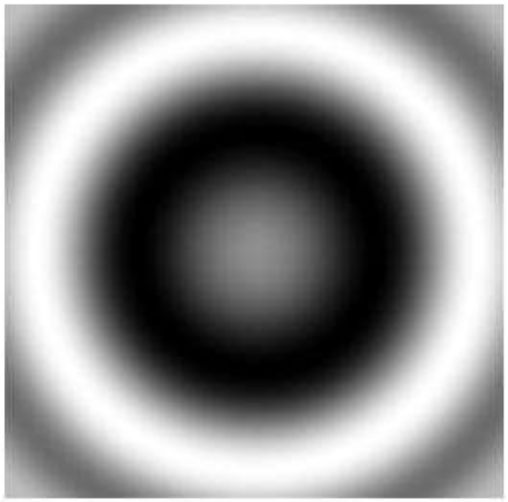
5. Averaged **power spectrum**



6. Least-squares fit between calculated and observed power spectrum

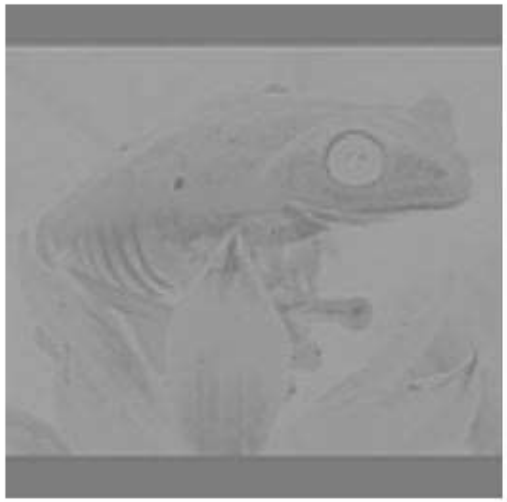
Real object

x



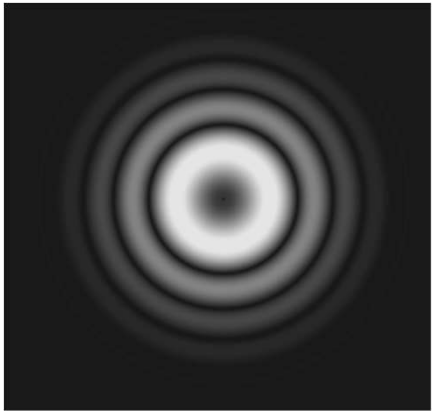
CTF

=



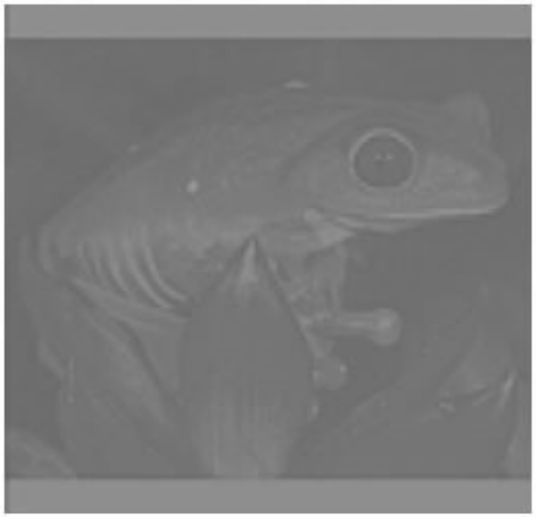
EM image

Phase flipping



A brutal correction of the CTF simply consists in flipping the function sign for frequencies in which $H(\boldsymbol{s}) < 0$, i.e. in black regions.

$$I(\boldsymbol{s}) = \begin{cases} I(\boldsymbol{s}) & \text{if} \quad H(\boldsymbol{s}) > 0 \\ -I(\boldsymbol{s}) & \text{if} \quad H(\boldsymbol{s}) < 0 \end{cases}$$



Wiener filter

Considering noise: $I(\boldsymbol{s}) = H(\boldsymbol{s}) \cdot O(\boldsymbol{s}) + N(\boldsymbol{s})$

Target:
$\hat{O}(\boldsymbol{s}) = S(\boldsymbol{s}) \cdot I(\boldsymbol{s})$

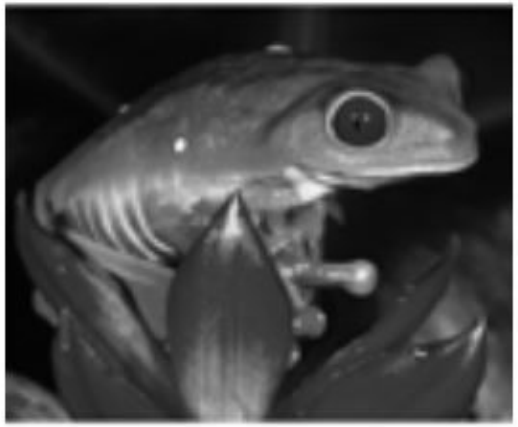
Wiener filter:

$$S(\boldsymbol{s}) = \frac{H^*(\boldsymbol{s})}{|H(\boldsymbol{s})|^2 + P_N(\boldsymbol{s})/P_I(\boldsymbol{s})}$$

$P_N(\boldsymbol{s})$: power spectrum of noise
$P_I(\boldsymbol{s})$: power spectrum of signal

If no noise is present: $\lim_{P_N \to 0} S(\boldsymbol{s}) = \frac{1}{|H(\boldsymbol{s})|}$

If there is only noise (no signal): $\lim_{P_I \to 0} S(\boldsymbol{s}) = 0$



Real object

Image collection issues

1. **Radiation damage**

   Present even at very low doses! ($< 10\ e^{-}/Å^{2}$)

   Slightly reduced by cooling the sample, but not sufficient

   For negatively stained samples, good contrast: dose can be reduced

   For cryo samples, poor contrast: AVERAGING!!

2. **Beam induced movements**

   Often induced by charging of the sample during data collection.

   Significantly reduces resolution of the image! (blurring)

   MOTION CORRECTION WITH DDD CAMERA!!

3. **Inelastic scattering**

   Increases noise level. Negligible for thin specimen, but important for thick samples, e.g. for tomography.

   ENERGY FILTER!!

References

- DDD camera: Li X. *et al.*, “Influence of electron dose rate on electron counting images recorded with the K2 camera.”, **J Struct Biol. 2013**, *184(2)*:251-60; Li X. *et al.*, “Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM.”, **Nat Methods. 2013**, *10(6)*:584-90; Chiu P.L. *et al.*, “Evaluation of super-resolution performance of the K2 electron-counting camera using 2D crystals of aquaporin-0. ”, **J Struct Biol. 2015**, *192(2)*:163-73.